



#4

SEQUENCE LISTING

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<120> METHODS FOR REGULATING A CELL-MEDIATED IMMUNE RESPONSE
BY BLOCKING LYMPHOCYTIC SIGNALS AND BY BLOCKING LFA-1
MEDIATED ADHESION

<130> D0009NP/30436.53USU1

<140> 09/877,987

<141> 2001-06-08

<150> 60/210,671

<151> 2000-06-09

<160> 9

<170> PatentIn Ver. 2.1

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Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	Ala	Leu
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ggcatcgcta	gctttgtgtg	tgagtatgca	tctccaggca	aataactga	ggccgggtg	180
acagtgttc	ggcaggctga	cagccagggtg	actgaagtct	gtgcggcaac	ctacatgatg	240
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Ala	Val	Val	Leu	Ala	Ser	Ser	Arg	Gly	Ile	Ala	Ser	Phe	Val	Cys	Glu
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Gln	Ala	Asp	Ser	Gln	Val	Thr	Glu	Val	Cys	Ala	Ala	Thr	Tyr	Met	Met
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Gly	Asn	Glu	Leu	Thr	Phe	Leu	Asp	Asp	Ser	Ile	Cys	Thr	Gly	Thr	Ser
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Thr	Ser	Pro	Pro	Ser	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Ser	Ser	Val
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Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr
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Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys
			245						250				255		
Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile
			260					265					270		
Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro
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Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg		
	340	345 350
Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu		
	355	360 365
His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys		
370	375	380



Exhibit 4

As used herein, "B7" refers to B7 family members including B7-1 (also known as CD80), B7-2 (also known as CD86) and B7-3 molecules that may recognize and bind CD28 and/or CTLA4.

5 As used herein "wild type CTLA4" has the amino acid sequence of naturally occurring, full length CTLA4 (U.S. Patent Nos. 5,434,131, 5,844,095, 5,851,795), or the extracellular domain thereof, which binds a B7, and/or interferes with a B7 from binding their ligands. In particular embodiments, the extracellular domain of wild type CTLA4 begins with methionine at position +1 and ends at aspartic acid at position +124, or the
10 extracellular domain of wild type CTLA4 begins with alanine at position -1 and ends at aspartic acid at position +124. Wild type CTLA4 is a cell surface protein, having an N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain. The extracellular domain binds to target antigens, such as a B7. In a cell, the naturally occurring, wild type CTLA4 protein is translated as an immature polypeptide,
15 which includes a signal peptide at the N-terminal end. The immature polypeptide undergoes post-translational processing, which includes cleavage and removal of the signal peptide to generate a CTLA4 cleavage product having a newly generated N-terminal end that differs from the N-terminal end in the immature form. One skilled in the art will appreciate that additional post-translational processing may occur, which
20 removes one or more of the amino acids from the newly generated N-terminal end of the CTLA4 cleavage product. The mature form of the CTLA4 molecule includes the extracellular domain of CTLA4, or any portion thereof, which binds to B7.

"CTLA4Ig" is a soluble fusion protein comprising an extracellular domain of wild type CTLA4, or a portion thereof that binds a B7, joined to an Ig tail. A particular
25 embodiment comprises the extracellular domain of wild type CTLA4 starting at methionine at position +1 and ending at aspartic acid at position +124; or starting at alanine at position -1 to aspartic acid at position +124; a junction amino acid residue glutamine at position +125; and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357 (Figure 5).

and SEQ ID NO: 6

Exhibit 4

<u>Amino Acid</u>	<u>Symbol</u>	<u>One Letter Symbol</u>	<u>Codons</u>
Histidine	His	H	CAU, CAC
Isoleucine	Ile	I	AUU, AUC, AUA
Lysine	Lys	K	AAA, AAG
Leucine	Leu	L	UUA, UUG, CUU, CUC, CUA, CUG
Methionine	Met	M	AUG
Asparagine	Asn	N	AAU, AAC
Proline	Pro	P	CCU, CCC, CCA, CCG
Glutamine	Gln	Q	CAA, CAG
Arginine	Arg	R	CGU, CGC, CGA, CGG, AGA, AGG
Serine	Ser	S	UCU, UCC, UCA, UCG, AGU, AGC
Threonine	Thr	T	ACU, ACC, ACA, ACG
Valine	Val	V	GUU, GUC, GUA, GUG
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAU, UAC

As used herein "the extracellular domain of CTLA4" is any portion of CTLA4 that recognizes and binds a B7. For example, an extracellular domain of CTLA4 comprises methionine at position +1 to aspartic acid at position +124 (Figure 5). Alternatively, an
 5 extracellular domain of CTLA4 comprises alanine at position -1 to aspartic acid at position +124 (Figure 5). The extracellular domain includes fragments or derivatives of CTLA4 that bind a B7. *an SEQ ID NO: 6* and *SEQ ID NO: 6*

As used herein, "lymphocyte" refers to mononuclear cells that mediate humoral- or cell-
 10 mediated immunity. Major subsets of lymphocytes include B and T cells.

As used herein, "immune system diseases" refer to autoimmune, immunoproliferative disorders and graft-related disorders including, but not limited to: graft-versus-host disease (GVHD) (e.g., such as may result from bone marrow transplantation, or in the
 15 induction of tolerance); immune disorders associated with graft transplantation rejection (e.g. chronic rejection, tissue or cell allo- or xenografts including solid organs, skin, islets,

disease being treated, the severity of the disease, a subject's health and response to the treatment with the agents. Accordingly, dosages of the agents can vary depending on each subject, agent and the mode of administration. For example, soluble CTLA4 molecules such as L104EA29YIg (included in Figure 6; as encoded by DNA deposited with ATCC accession number PTA-2104; and as described in U.S. Patent Application Serial Numbers 09/579,927, 60/287,576 and 60/214,065, incorporated by reference herein), may be administered in an amount between 0.1 to 20.0 mg/kg weight of a human subject/day, preferably between 0.5 to 10.0 mg/kg/day. and SEQ ID NOs: 8 and 9

Administration of the agents may be performed in many permissible ways including, but not limited to: injection (e.g. intravenous, intraperitoneal, intramuscular, etc.), oral administration, inhalation, topical contact, gene therapy, administration by a mechanical release device such as a pump, administration of slow release devices such as vesicles or capsules, or suppositories. Depending on the means of administration, the agents may be compounded with pharmaceutically acceptable carriers for convenient application and effective use of the agents.

The pharmaceutical compositions also preferably include suitable carriers and adjuvants which include any material which when combined with the molecule of the invention (e.g., a soluble CTLA4 mutant molecule, such as, L104EA29Y or L104E) retains the molecule's activity and is non-reactive with the subject's immune system. Examples of suitable carriers and adjuvants include, but are not limited to, human serum albumin; ion exchangers; alumina; lecithin; buffer substances, such as phosphates; glycine; sorbic acid; potassium sorbate; and salts or electrolytes, such as protamine sulfate. Other examples include any of the standard pharmaceutical carriers such as a phosphate buffered saline solution; water; emulsions, such as oil/water emulsion; and various types of wetting agents. Other carriers may also include sterile solutions; tablets, including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

A nucleotide sequence encoding CTLA4Ig was first generated as described *infra*, then a single-site mutant L104EIg was derived from the CTLA4Ig sequence and tested for binding kinetics for CD80 and/or CD86. The L104EIg nucleotide sequence was used as a template to generate the double-site mutant CTLA4 sequence, L104EA29YIg, which
 5 was tested for binding kinetics for CD80 and/or CD86.

Construction of CTLA4Ig

A genetic construct encoding CTLA4Ig comprising the extracellular domain of CTLA4 and
 10 an IgCgamma1 domain was constructed as described in U.S. Patents 5,844,095 and 5,851,795, the contents of which are incorporated by reference herein. The extracellular domain of the CTLA4 gene was cloned by PCR using synthetic oligonucleotides corresponding to the published sequence as described by Dariavach et al. (72).

(SEQ ID NO:1)

15 Because a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N-terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (73) in two steps using overlapping oligonucleotides. For the first step, the oligonucleotide, CTCAGTCTGGTCCTTGCACCTCCTGTTTCCAAGCATGGCGAGCA
 TGGCAATGCACGTGGCCCAGCC (which encoded the C terminal 15 amino acids
 20 from the oncostatin M signal peptide fused to the N terminal 7 amino acids of CTLA4) was used as forward primer, and TTTGGGCTCCTGATCAGAATCTGGGCACGGTTG

(SEQ ID NO:2) (encoding amino acid residues 119-125 of the amino acid sequence encoding CTLA4 receptor and containing a Bcl I restriction enzyme site) as reverse primer. The template for this step was cDNA synthesized from 1 micro g of total RNA from H38 cells (an
 25 HTLV II infected T-cell leukemic cell line provided by Drs. Salahudin and Gallo, NCI, Bethesda, MD). A portion of the PCR product from the first step was reamplified, using an overlapping forward primer, encoding the N terminal portion of the oncostatin M signal peptide and containing a Hind III restriction endonuclease site, CTAGCCACTGAAGCTTCACCAATGGGTGTACTGCTCACACAGAGGACGCTGC
 30 TCAGTCTGGTCCTTGCACCTC and the same reverse primer. The product of the PCR reaction was digested with Hind III and Bcl I and ligated together with a Bcl I/Xba I

(SEQ ID NO:3)

cleaved cDNA fragment encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of IgG1 into the Hind III/Xba I cleaved expression vector, CDM8 or Hind III/Xba I cleaved expression vector piLN (also known as π LN).

5 DNA encoding the amino acid sequence corresponding to CTLA4Ig has been deposited with the ATCC under the Budapest Treaty on May 31, 1991, and has been accorded ATCC accession number 68629.

CTLA4Ig Codon Based Mutagenesis to generate double mutants:

10 A mutagenesis and screening strategy was developed to identify mutant CTLA4Ig molecules that had slower rates of dissociation ("off" rates) from CD80 and/or CD86 molecules. Single-site mutant nucleotide sequences were generated using CTLA4Ig (U.S. Patent Numbers: 5,844,095; 5,851,795; and 5,885,796; ATCC Accession No. 68629) as a template. Mutagenic oligonucleotide PCR primers were designed for random
15 mutagenesis of a specific cDNA codon by allowing any base at positions 1 and 2 of the codon, but only guanine or thymine at position 3 (XXG/T; also known as NNG/T). In this manner, a specific codon encoding an amino acid could be randomly mutated to code for each of the 20 amino acids. In that regard, XXG/T mutagenesis yields 32 potential codons encoding each of the 20 amino acids. PCR products encoding mutations in close
20 proximity to -M97-G107 of CTLA4Ig (see Figure 5), were digested with SacI/XbaI and subcloned into similarly cut CTLA4Ig π LN expression vector. This method was used to generate the single-site CTLA4 mutant molecule L104EIg. and SEQ ID Nos: 6 and 7

For mutagenesis in proximity to S25-R33 of CTLA4Ig, a silent NheI restriction site was
25 first introduced 5' to this loop, by PCR primer-directed mutagenesis. PCR products were digested with NheI/XbaI and subcloned into similarly cut CTLA4Ig or L104EIg expression vectors. This method was used to generate the double-site CTLA4 mutant molecule L104EA29YIg (Figure 6). In particular, the nucleic acid molecule encoding the single-site CTLA4 mutant molecule, L104EIg, was used as a template to generate the
and SEQ ID Nos: 8 and 9

double-site CTLA4 mutant molecule, L104EA29YIg. The sequence of L104EA29YIg is shown in Figure 6 and includes an N-terminal leader sequence.

and SEQ ID NOS: 8 and 9

5 The following provides a description of the screening methods used to identify the single- and double-site mutant CTLA4 polypeptides, expressed from the constructs described *supra*, that exhibited a higher binding avidity for CD80 and CD86 antigens, compared to non-mutated CTLA4Ig molecules.

10 Current *in vitro* and *in vivo* studies indicate that CTLA4Ig by itself is unable to completely block the priming of antigen specific activated T cells. *In vitro* studies with CTLA4Ig and either monoclonal antibody specific for CD80 or CD86 measuring inhibition of T cell proliferation indicate that anti-CD80 monoclonal antibody did not augment CTLA4Ig inhibition. However, anti-CD86 monoclonal antibody did augment the inhibition, indicating that CTLA4Ig was not as effective at blocking CD86
15 interactions. These data support earlier findings by Linsley et al. (74) showing inhibition of CD80-mediated cellular responses required approximately 100 fold lower CTLA4Ig concentrations than for CD86-mediated responses. Based on these findings, it was surmised that soluble CTLA4 mutant molecules having a higher avidity for CD86 than wild type CTLA4 should be better able to block the priming of antigen specific activated
20 cells than CTLA4Ig.

To this end, the soluble CTLA4 mutant molecules described above were screened using a novel screening procedure to identify several mutations in the extracellular domain of CTLA4 that improve binding avidity for CD80 and CD86. This screening strategy
25 provided an effective method to directly identify mutants with apparently slower "off" rates without the need for protein purification or quantitation since "off" rate determination is concentration independent as described by O'Shannessy et al (75).

COS cells were transfected with individual miniprep purified plasmid DNA and propagated for several days. Three day conditioned culture media was applied to
30 BIAcore biosensor chips (Pharmacia Biotech AB, Uppsala, Sweden) coated with soluble

Flow Cytometry:

Murine mAb L307.4 (anti-CD80) was purchased from Becton Dickinson (San Jose, California) and IT2.2 (anti-B7-0 [also known as CD86]), from Pharmingen (San Diego, California). For immunostaining, CD80-positive and/or CD86-positive CHO cells were removed from their culture vessels by incubation in phosphate-buffered saline (PBS) containing 10mM EDTA. CHO cells ($1-10 \times 10^5$) were first incubated with mAbs or immunoglobulin fusion proteins in DMEM containing 10% fetal bovine serum (FBS), then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-human immunoglobulin second step reagents (Tago, Burlingame, California). Cells were given a final wash and analyzed on a FACScan (Becton Dickinson).

SDS-PAGE and Size Exclusion Chromatography

SDS-PAGE was performed on Tris/glycine 4-20% acrylamide gels (Novex, San Diego, CA). Analytical gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning. CTLA4Ig (25 μ g) and L104EA29YIg (25 μ g) were analyzed by size exclusion chromatography using a TSK-GEL G300 SW_{XL} column (7.8 x 300mm, Tosohaas, Montgomeryville, PA) equilibrated in phosphate buffered saline containing 0.02% NaN₃ at a flow rate of 1.0 ml/min.

CTLA4X_{C120S} and L104EA29YX_{C120S}.

Single chain CTLA4X_{C120S} was prepared as previously described (Linsley et al., (1995) J. Biol. Chem., 270:15417-15424 (84)). Briefly, an oncostatin M CTLA4 (OMCTLA4) expression plasmid was used as a template, the forward primer, GAGGTGATAAAGCTTCACCAATGGGTGTACTGCTCACACAG (SEQ ID No: 4) was chosen to match sequences in the vector; and the reverse primer, GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC (SEQ ID No: 5) corresponded to the last seven amino acids (i.e. amino acids 118-124) in the extracellular domain of CTLA4, and contained a restriction enzyme site, and a stop codon (TGA). The reverse primer specified a C120S (cysteine to serine at position 120) mutation. In particular, the nucleotide sequence GCA (nucleotides 34-36) of the reverse primer shown